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Burkholderia cenocepacia C5424 Produces a Pigment with Antioxidant Properties Using a Homogentisate Intermediate▽

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Burkholderia cenocepacia **is a gram-negative opportunistic pathogen that belongs to the *Burkholderia cepacia* complex. *B. cenocepacia* can survive intracellularly within phagocytic cells, and some epidemic strains produce a brown melanin-like pigment that can scavenge free radicals, resulting in the attenuation of the host cell oxidative burst. In this work, we demonstrate that the brown pigment produced by *B. cenocepacia* C5424 is synthesized from a homogentisate (HGA) precursor. The disruption of BCAL0207 (*hppD*) by insertional inactivation resulted in loss of pigmentation. Steady-state kinetic analysis of the BCAL0207 gene product demonstrated that it has 4-hydroxyphenylpyruvic acid dioxygenase (HppD) activity. Pigmentation could be restored by complementation providing *hppD* in *trans*. The *hppD* mutant was resistant to paraquat challenge but sensitive to H_2O_2 and to extracellularly generated superoxide anions. Infection experiments in RAW 264.7 murine macrophages showed that the nonpigmented bacteria colocalized in a dextran-positive vacuole, suggesting that they are being trafficked to the lysosome. In contrast, the wild-type strain did not localize with dextran. Colocalization of the nonpigmented strain with dextran was reduced in the presence of the NADPH oxidase inhibitor diphenyleneiodonium, and also the inducible nitric oxide inhibitor aminoguanidine. Together, these observations suggest that the brown pigment produced by *B. cenocepacia* C5424 is a pyomelanin synthesized from an HGA intermediate that is capable of protecting the organism from in vitro and in vivo sources of oxidative stress.**

Melanin is a negatively charged hydrophobic macromolecule of high molecular weight. The exact structure of melanin is unknown, but it is formed by the oxidative polymerization of phenolic and/or indolic compounds and contains a stable population of organic free radicals (42). The resulting pigments are usually brown or black. Melanin-like pigments can be produced by a variety of microorganisms, including fungi, bacteria, and helminths (37). Melanins can be divided into four classes: eumelanins, phaeomelanins, allomelanins, and pyomelanins. Eumelanins are derived from quinines and free radicals, phaeomelanins are derived from tyrosine and cysteine, allomelanins are formed from nitrogen-free precursors, and pyomelanins are derived from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate and homogentisate (HGA) (13). The bacterial genera *Aeromonas*, *Legionella*, *Streptomyces*, *Pseudomonas*, *Bacillus*, *Vibrio*, *Hyphomonas*, and *Shewanella* produce melanin-like pigments (3, 6, 20, 26, 62). Of these, all but *Aeromonas* and *Bacillus* synthesize a pyomelanin from an HGA intermediate (2, 8, 26). The ability of a microorganism to produce melanin has been linked with pathogenicity and virulence for their respective plant or animal hosts (reviewed in references 37 and 38).

HGA biosynthesis includes a decarboxylation step, dioxygenation, and the rearrangement of a pyruvate side chain (31, 46, 47). This complex reaction is carried out by the 4-hydroxyphenylpyruvic acid dioxygenase (HppD; EC 1.13.11.27), a nonheme iron-dependent enzyme that is active as a homotetramer in bacteria and as a homodimer in plants. HppD has been described in humans (4, 44), mouse (9), and rat (10), as well as plants (14, 16), fungi (63), and prokaryotes (8, 45). There is considerable interest in the HGA catabolic pathway, because HppD in plants is an important herbicide target (5, 15, 23) and many severe human diseases, like phenylketonuria (18); alkaptonuria (52); tyrosinemias I, II, and III; and hawkinsinuria (48), are associated with enzyme deficiencies in the catabolism of tyrosine.

The gram-negative bacterium *Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (Bcc) (59). Bcc members, particularly *B. cenocepacia* and *Burkholderia multivorans*, cause opportunistic infections in patients suffering from chronic granulomatous disease and cystic fibrosis (CF) (35, 56). Once established in the lung of a CF patient, Bcc infection is rarely eradicated and is often associated with persistent inflammation, rapid decay of lung function, and, in some cases, a sepsis-like syndrome known as "cepacia syndrome" (17, 21, 58). During *B. cenocepacia* colonization and infection, the airways of CF patients exhibit a pronounced inflammatory response that results in the release of reactive oxygen and reactive nitrogen species. Previous research using *B. cenocepacia* strain P1 (Cardiff epidemic strain) demonstrated that the strain could produce a brown melanin-like pigment that was capable of attenuating the oxidative burst of the human monocyte cell line MonoMac-6 (64).

In this study, we report the identification of HGA as an essential precursor for the production of a melanin-like pigment in *B. cenocepacia* strain C5424. A strain harboring a

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F$^-$ ϕ80 *lacZ* ΔM15 Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* Δ*gyrA96 relA1*	Laboratory stock
SY327	*araD* Δ(*lac* pro) *argE*(Am) *recA56 rifr nalA*, λ *pir*	Laboratory stock
B. cenocepacia		
C5424	Clinical isolate	BCRRC[b]
LEK47	C5424; *hppD*::pLK2 Tp^r	This study
K56-2	Clinical isolate	BCRRC
Plasmids		
pGPΩTp	ori_{R6K} Tp^r	12
pDA17	$ori_{pBBR1}P_{DHFR}$ Tet^r	Aubert and Valvano (unpublished)
pLK2	pGP-ΩTp; 300-bp *hppD* mutagenesis fragment	This study
pKK29	pDA17; $hppD_{Flag}$	This study
pKK50	pET28a; *hppD* His_6	This study
pRK2013	RK2 derivative; ori_{colE1} Km^r *mob*$^+$ *tra*$^+$	11

[a] Cm, chloramphenicol; Km, kanamycin; Tet, tetracycline; Tp, trimethoprim.
[b] BCRRC, *B. cepacia* Complex Research and Referral Repository for Canadian CF Clinics.

mutation in the BCAL0207 gene, which encodes an HppD homologue, was created, and the disruption of this gene, herein designated *hppD*, resulted in a nonpigmented strain. The absence of pigment led to increased sensitivity to oxidative stress in vitro and reduced bacterial intracellular survival in a murine macrophage cell line.

MATERIALS AND METHODS

Reagents, bacterial strains, macrophage cell line, and culture conditions. Chemicals and reagents used in this study were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise stated. The sodium salt of HEPES buffer, 4-hydroxyphenylpyruvic acid (HPP), and iron(II) ammonium sulfate were purchased from ACROS, and dithiothreitol was from Gold Biotechnology Inc. Bacterial strains and plasmids are described in Table 1. *Escherichia coli* and *B. cenocepacia* strains were grown at 37°C in Luria broth (LB). Trimethoprim (50 μg/ml for *E. coli* and 100 μg/ml for *B. cenocepacia*) and tetracycline (20 μg/ml for *E. coli* and 100 μg/ml for *B. cenocepacia*) were added as appropriate. Gentamicin (50 μg/ml) was used during triparental-mating experiments. Bacterial growth was measured by monitoring the optical density at 600 nm in triplicate cultures. The murine macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection, Manassas, VA, and routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Wisent Inc., St. Bruno, Quebec, Canada).

Pigment production. *B. cenocepacia* strains were streaked heavily on LB agar plates, incubated for 36 to 48 h at 37°C, and observed for pigment production.

Bioinformatic analysis. BLAST-X searches of the *B. cenocepacia* strain J2315 genome were performed using the nucleotide sequences of *hppD* genes from other gram-negative organisms as the query sequences. Putative HppD proteins were then screened for the presence of HppD motifs using the PROSITE protein families and motifs database (http://www.expasy.org/prosite/).

PCR amplifications. PCR amplifications were performed in a PTC-0200 DNA engine (MJ Research) using either *Pwo* polymerase (Roche) or *Taq* polymerase (Qiagen), the supplied Q solution for G+C-rich templates, and Bcc chromosomal DNA as a template. The specific PCR conditions were optimized for each primer pair. PCR amplification products were separated on 0.7% agarose gels and purified using the QiaQuick gel extraction kit according to the manufacturer's instructions (Qiagen).

Construction of an *hppD* insertional mutant of *B. cenocepacia* C5424. pGPΩTp, a derivative of pGP704 that carries the Pir-dependent R6K origin of replication and the *dhfr* gene flanked by terminator sequences, was used to disrupt *hppD*. A 300-bp internal fragment of the *hppD* gene of *B. cenocepacia* C5424 was amplified by PCR using primers 2379 (5′-AAAATCTAGAGTCGG CACCGACGGCTTC-3′) and 2380 (5′-AAAATCTAGAGGATGTTCAGCTC CATCGGG-3′) (XbaI recognition sites are underlined). The product was ligated into the XbaI site of pGPΩTp and transformed into *E. coli* SY327. Trimethoprim-resistant colonies were screened by restriction digestion and PCR using primers 1300 (5′-TAACGGTTGTGGACAACAAGCCAGGG-3′) and 2379 to confirm the presence and orientation of the *hppD* internal fragment. The plasmid pLK2, which contained the *hppD* internal fragment, was transferred to *B. cenocepacia* C5424 by triparental mating (7). Exconjugants that had pLK2 integrated into the C5424 genome were selected on LB agar supplemented with trimethoprim and gentamicin (to remove *E. coli* helper and donor strains). The integration of the suicide plasmid was confirmed by PCR using primers 1300 (5′-TAACGGTTGTGGACAACAAGCCAGGG-3′) and 2345 (5′-AAAACCA TGGATGCAGATCCCCACCTGGGACA-3′) and Southern blot hybridization using an *hppD*-specific probe, allowing the identification of the *hppD*-deficient strain LEK47.

Southern blot hybridization analysis. The 300-bp amplicon (*hppD*) probe was labeled directly with digoxigenin-11-UTP using primers 2379 and 2380 and a PCR labeling kit (Roche), as recommended by the manufacturer. *B. cenocepacia* genomic DNA was isolated and digested with XhoI. Briefly, the DNA was separated by electrophoresis on a 0.7% agarose gel and transferred to a nitrocellulose membrane by capillary action. The membrane was incubated with the *hppD* probe under high-stringency conditions. Hybridization signals were detected by chemiluminescence with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricylco[3.3.1.1^{3,7}]decan}-4yl)phenyl phosphate as recommended by the manufacturer (Roche).

Complementation of the *hppD* mutant. A PCR fragment carrying the complete coding sequence of the *hppD* gene was amplified from *B. cenocepacia* C5424 chromosomal DNA using the forward primer 2514 (5′-AAAAACATATGCAG ATCCCCACCTGGGACAACC-3′) and the reverse primer 2515 (5′-AAAATC TAGAGGCCTTGTCCTGCACGACGC-3′) containing NdeI and XbaI restriction sites, respectively (underlined). The *hppD* PCR product was digested with NdeI and XbaI and ligated into NdeI- and XbaI-digested pDA17 before transformation into *E. coli* DH5α. The resulting plasmid, pKK29, encoded an HppD protein with a C-terminal FLAG epitope ($HppD_{FLAG}$). This was verified by colony PCR using primers 1631 (5′-ACTCTCGCATGGGGAGACCC-3′) and 2514, restriction digestion, and DNA sequencing (by the York University Core Molecular Biology and DNA Sequencing Facility, Toronto, Ontario, Canada), confirming the presence of the insert and that no mutations were present in the PCR-amplified *hppD* sequence compared to the published strain J2315 sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Chemical complementation was also performed by the addition of 0.5 mM homogentisic acid (Sigma-Aldrich) to the LEK47 growth medium.

Identification of HGA in culture supernatants by high-performance liquid chromatography (HPLC). A Waters 2695 Alliance high-performance liquid chromatograph with a 996 photodiode array detector and a Nova-Pak C_{18} column (length, 150 mm; inside diameter, 3.9 mm) was used to analyze culture

supernatant samples. Supernatants (1 ml) were mixed with 100 μl of glacial acetic acid, clarified by centrifugation, and then stored at −20°C until they were assayed. The frozen samples were thawed, diluted threefold with 10 mM acetic acid, and then filtered with a 0.45-μm filter; 20 μl of culture supernatant was injected on the Nova-Pak column and eluted at a flow rate of 0.85 ml/min. The mobile phase was 10 mM acetic acid-methanol (90:10 [vol/vol]). The wavelength was set to 290 nm, as previously described (8). The peak corresponding to HGA was identified by comparison of the chromatograms of standard solutions of HGA. The spectrum of HGA had an absorption maximum at 290 nm.

In vitro sensitivity to extracellular superoxide. Assays were performed using a xanthine/xanthine oxidase system to generate extracellular superoxide (50). Late-stationary-phase culture samples containing 1×10^8 cells ml^{-1} were incubated with shaking at 37°C in a mixture containing 250 μM xanthine and 0.14 units of xanthine oxidase. Catalase (100 U ml^{-1}) was added to each sample prior to the addition of xanthine oxidase to protect the cells from the toxicity of any H_2O_2 produced as a consequence of the superoxide dismutase activity (24). Aliquots were removed at 0, 30, 60, and 120 min and serially diluted in 1× phosphate-buffered saline, pH 7.4. Time zero aliquots were removed before the addition of xanthine oxidase. Appropriate dilutions were plated in triplicate on LB agar plates and incubated overnight at 37°C. Percentage survival was calculated as described previously (30).

Disc diffusion assays. Late-stationary-phase cells were spread on agar plates with a sterile cotton swab, and 6-mm sterile paper discs were applied to the surfaces. Eight-microliter volumes of 0 to 100 mM H_2O_2 or 0 to 10 mM methyl viologen (paraquat) were applied to triplicate discs. The plates were incubated overnight at 37°C, and zones of inhibition were measured.

Biochemical characterization. A 50-ml culture of *B. cenocepacia* C5424 was grown in LB for 48 h at 37°C until it was heavily pigmented, and the supernatant was isolated by centrifugation at 8,000 × *g* for 20 min. The pigment was precipitated by the addition of ethanol to a final concentration of 66% (64). The solubility of the precipitated pigment was tested with alkaline water (pH 13). Bleaching experiments with 30% (wt/wt) H_2O_2 and NaOCl were also performed (1).

Cloning *B. cenocepacia hppD* into pET28a. The *B. cenocepacia* C5424 *hppD* gene was amplified by PCR using primers 2999 (5′-AAAACTCGAGTCAGTC CTGCACGACGCCGC-3′) and 3008 (5′-AAAACATATGCAGATCCCCACC TGGGACAAC-3′), including NdeI and XhoI restriction sites; ligated into NdeI- and XhoI-digested pET28a; and transformed into *E. coli* DH5α cells, creating pKK50. Kanamycin-resistant colonies were screened by restriction digestion and PCR to confirm the presence of *hppD*. pKK50 was confirmed by DNA sequencing using T7 promoter and terminator primers specific for the pET vectors.

Overexpression and purification of *B. cenocepacia* HppD in *E. coli* BL21(DE3). pKK50 was transformed into *E. coli* BL21(DE3). A single colony was then used to inoculate four 5-ml volumes of LB plus kanamycin, and the culture was grown overnight with shaking at 37°C. Three liters of LB plus kanamycin was then inoculated 1:100 with the overnight culture and incubated until the cells reached an optical density at 600 nm of 0.6 to 0.8. The cells were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.5 mM and allowed to grow for a further 4 h before being harvested by centrifugation at 8,000 × *g* and 4°C for 15 min. The cell pellets were resuspended in 1.5% of the original culture volume of cell lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), 0.75 mg/ml lysozyme was added, and the suspension was incubated for 20 min at room temperature. The cells were lysed by sonic disruption, and soluble proteins were then harvested by centrifugation at 8,000 × *g* for 20 min at 4°C and filtered through a 0.45-μm filter. Soluble proteins were applied to a 5-ml-bed-volume Hi-Trap chelating HP column charged with cobalt ions and equilibrated with cell lysis buffer as recommended by the manufacturer. The column was washed with 5 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The proteins were then eluted in 4 column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Elution fractions containing recombinant *B. cenocepacia* HppD were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining. These fractions were concentrated using a Vivaspin centrifugal concentrator with a 10-kDa cutoff. The HppD was further purified using a Superdex 200 10/300 GL size exclusion column, and 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl was used as a mobile phase; 0.5-ml fractions were collected, fractions containing HppD were pooled, and the protein concentration was determined by Bradford assay.

Enzyme assay and steady-state kinetics. The HppD activity was measured using a DW1 Hansatech Oxygraph dioxygen electrode. The molar extinction coefficient of *B. cenocepacia* HppD was calculated to be 41,000 M^{-1} cm^{-1} at 280 nm (39). The molar extinction coefficient of HPP was 3,400 M^{-1} cm^{-1} at 276 nm at pH 7.0 (22). Assay mixtures included 0.5 M of enzyme, 1 mM dithiothreitol, 10 μM Fe(II), and HPP in 20 mM HEPES, pH 7.0, at 25°C with atmospheric oxygen (~250 M). Reactions were initiated with HPP, and the rates were measured from the rate that occurred between 20 and 50 seconds of turnover. Apparent kinetic parameters were obtained by measuring the rate of dioxygen consumption in assays with varied HPP concentrations. Data were fitted to the Michaelis-Menten equation using Kaleidagraph software (Synergy Software, Reading, PA).

Macrophage infections. Cell culture reagents were purchased from Wisent Inc., St. Bruno, Quebec, Canada, unless otherwise stated. Macrophages were trypsinized and seeded into six-well tissue culture plates containing glass coverslips. The cells were incubated overnight at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. Labeling of the endosomal pathway was performed by incubating the macrophages with dextran tetramethylrhodamine (10,000 molecular weight) (TMR-dextran) at a final concentration of 250 μg/ml for 2 h at 37°C. The external TMR-dextran was then removed by washing the macrophages three times with prewarmed phosphate-buffered saline, and then the medium was refreshed and bacteria were added. Bacteria were grown for 36 h (until pigment was produced by C5424) and then washed twice with DMEM; the RAW 264.7 macrophage-like cells were then infected with either C5424 or LEK47 at a multiplicity of infection of 30. Infections were equalized by centrifugation at 1,500 rpm for 1 min and were allowed to proceed for 2 h. After this period, the external bacteria were removed by washing the cultures three times with RPMI prewarmed to 37°C. In some experiments, 10 μM diphenyleneiodonium (DPI) or 100 μM aminoguanidine (AG) was added concurrently with the addition of bacteria to the macrophages. Fluorescence and phase-contrast images of the infected macrophage monolayers were then acquired using a Qimaging (Burnaby, British Columbia, Canada) cooled charged-coupled-device camera on an Axioscope 2 microscope (Carl Zeiss, Thornwood, NY) with a 100×/1.3-numerical-aperture Plan-Neofluor objective and a 50-W mercury arc lamp. Red filter set 15 (Carl Zeiss, Thornwood, NY) with short-pass 546-nm excitation and low-pass 590-nm emission was used. Images were digitally processed using the Northern Eclipse version 6.0 imaging analysis software (Empix Imaging, Mississauga, Ontario, Canada).

RESULTS AND DISCUSSION

Molecular cloning of *B. cenocepacia* C5424 *hppD*. The production of a brown melanin pigment was described in a number of *B. cenocepacia* strains, including the genome sequence strain J2315 and the Cardiff epidemic strain P1 (64). However, nothing is known about the mechanism of melanin biosynthesis in *B. cenocepacia*. The production of bacterial melanin pigments usually occurs through the catabolism of tyrosine via either an HGA or 3,4-dihydroxyphenylpyruvate (DOPA) intermediate (Fig. 1B). *P. putida*, a gammaproteobacterium related to *B. cenocepacia*, synthesizes a melanin-like pigment via an HGA intermediate (2). Analysis of the *B. cenocepacia* J2315 genome resulted in the identification of an open reading frame on chromosome 1 (BCAL0207; *hppD*) that encodes a putative HppD. The putative HppD protein shares 63%, 57%, and 32% identity with HppD proteins from *Aeromonas hydrophila*, *Pseudomonas fluorescens*, and *Streptomyces avermitilis*, respectively. We hypothesized that the *B. cenocepacia hppD* gene product catalyzes the conversion of 4-hydroxyphenylpyruvate to HGA, which in turn is further oxidized and polymerized, producing the characteristic brown melanin pigment. Figure 1A shows the genetic organization of the area surrounding *hppD*, which differs from that found in other gammaproteobacteria, including, *Pseudomonas putida*, *P. fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Azotobacter vinelandii*, and *Xanthomonas axonopodis* (reviewed in references 2 and 19). In these organisms, *hppD* is in gene clusters encoding either the peripheral or the central pathway for phenylalanine and tyrosine catabolism. In contrast, bioinformatic analysis of this region revealed that BCAL0207 does not appear to be part of an operon, nor is it associated with genes involved in phe-

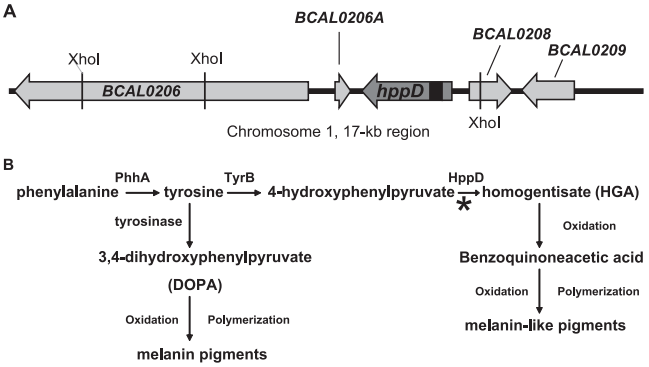

FIG. 1. (A) Genetic organization of the 17-kb region surrounding *hppD* in *B. cenocepacia* J2315. Annotation of the putative open reading frames (gray) on chromosome 1 that border *hppD*. BCAL0206 encodes a conserved hypothetical protein, BCAL0206A includes a possible gene remnant that is homologous to the N terminus of *motA* from *C. crescentus*, BCAL0208 encodes a putative AsnC family transcriptional regulator, and BCAL0209 encodes a conserved hypothetical protein. The internal fragment of *hppD* that was used to introduce the suicide plasmid pGPΩTp is indicated in black. (B) Two alternative pathways utilized by bacteria in the production of melanin pigments, via either a DOPA or an HGA intermediate. The asterisk indicates the location of the HppD gene mutated in this study.

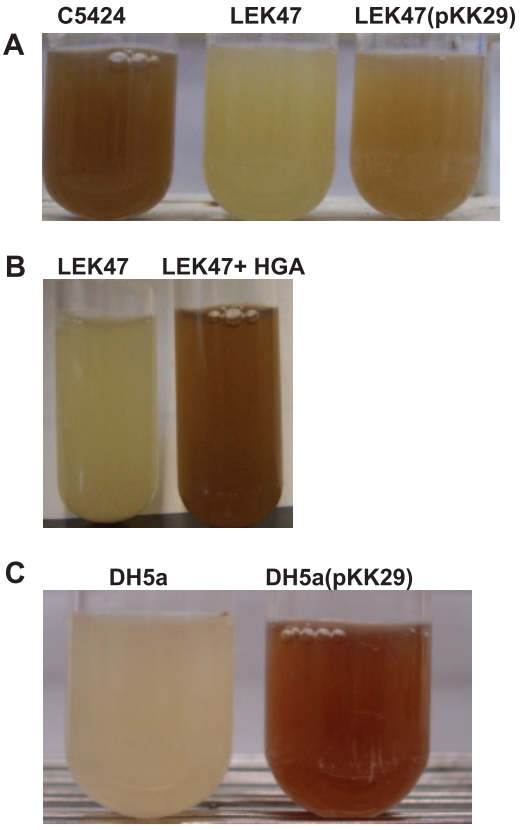

FIG. 2. (A) The production of pigment in LB media is abrogated in the *hppD* mutant LEK47. Pigmentation can be restored by the addition of the plasmid pKK29. (B) Pigment production can be restored to LEK47 by the addition of 0.5 mM HGA to the culture medium. (C) The nonpigmented strain *E. coli* DH5α produces a brown melanin-like pigment when transformed with pKK29.

nylalanine and tyrosine catabolism. Upstream are BCAL0208 and BCAL0209, encoding a putative AsnC family transcriptional regulator and a conserved hypothetical protein, respectively. Downstream are BCAL0206A and BCAL0206, encoding a conserved hypothetical protein and a pseudogene homologous to the N-terminal region of *motA* from *Caulobacter crescentus*, respectively. Therefore, the *B. cenocepacia hppD* gene described here is unique in that it is not associated with genes involved in either central or peripheral pathways for phenylalanine and tyrosine catabolism. The *B. cenocepacia* genome-sequencing strain J2315 belongs to the ET12 lineage, which also includes the clonally related strains K56-2, BC7, and C5424 (33). In this study, we utilized strain C5424 because it is much more amenable to genetic manipulation and naturally produces more pigment than strain J2315. The *hppD* gene from C5424 was 99% identical at the DNA sequence level to *hppD* from J2315 and 100% identical at the amino acid level; the gene organization also appears to be conserved in the surrounding region. The genome sequence for strain C5424 is not available. However, analysis of the J2315 genome revealed the presence of putative *hmgA* and *hmgB* homologues (BCAL3184 and BCAL3183, respectively). A homologue of tyrosine transaminase (BCAL2303) that would putatively convert phenylalanine into HPP was also identified.

Disruption of *hppD* results in a nonpigmented *B. cenocepacia* C5424 strain. Melanin is a substance with a dark color that is insoluble in aqueous or organic solvent, resistant to concentrated acid, and susceptible to bleaching by oxidizing agents (37, 38). The pigment produced by *B. cenocepacia* C5424 is dark brown, is soluble in alkaline water, can be precipitated by the addition of ethanol (64), and is susceptible to bleaching by both H_2O_2 and NaOCl (1). Taken together, these properties suggest that the C5424 pigment is melanic in nature. To evaluate whether the brown melanin pigment produced by *B. cenocepacia* C5424 is synthesized from an HGA intermediate, a *B. cenocepacia* mutant defective in the putative *hppD* gene was constructed and named LEK47. This mutant is an isogenic derivative of C5424 with an insertional inactivation of *hppD* by the integration of the suicide plasmid pLK2. The integration of pLK2 into the *B. cenocepacia* C5424 chromosome was confirmed by colony PCR and Southern blotting (data not shown). No pigment production was observed when LEK47 was cultured on LB plates and in LB media for 72 h, while under the same conditions, the wild-type strain, C5424, produced significant amounts of pigment by 36 h (Fig. 2A), and the level of pigment produced increased with time (data not shown). Pigment production could be restored to LEK47 by supplying the *hppD* gene in *trans* on the plasmid pKK29 under the control of the constitutive *dhfr* promoter (Fig. 2A). Expression of *B. cenocepacia hppD* from pKK29 also resulted in the production of a brown melanin-like pigment in the nonpigmented *E. coli* DH5α (Fig. 2C). Overexpression of *hppD* genes from *Arabidopsis* sp. strain PRL2, *Mycosphaerella graminicola*, *S. avermitilis*, and *Legionella pneumophila* in *E. coli* has also been shown to mediate production of a melanin-like pigment in *E. coli* (8, 25, 36, 57).

Since disruption of *hppD* would result in the loss of HppD activity and abrogation of HGA production, the absence of pigmentation in the mutant LEK47 suggests the pigment was

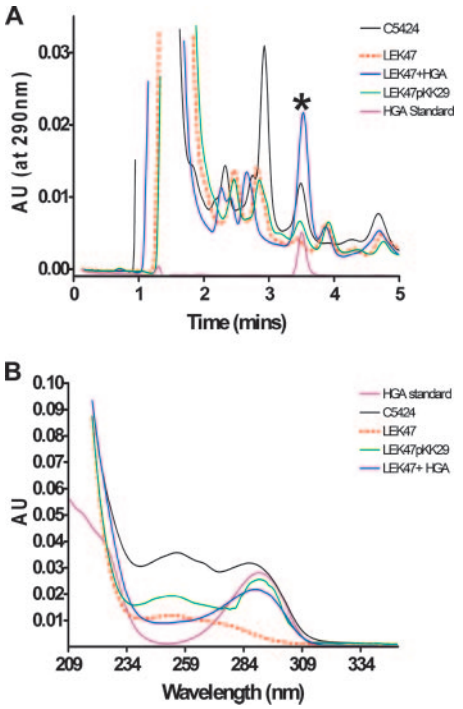

FIG. 3. HPLC chromatograms of culture supernatants. Samples were taken after 36 h of growth at 37°C in LB. (A) The asterisk indicates a 3.5-min peak that corresponds to the retention time exhibited by pure HGA, used as a standard, when run under the same conditions as the experimental samples. (B) Spectra obtained from the 3.5-min peak from each sample.

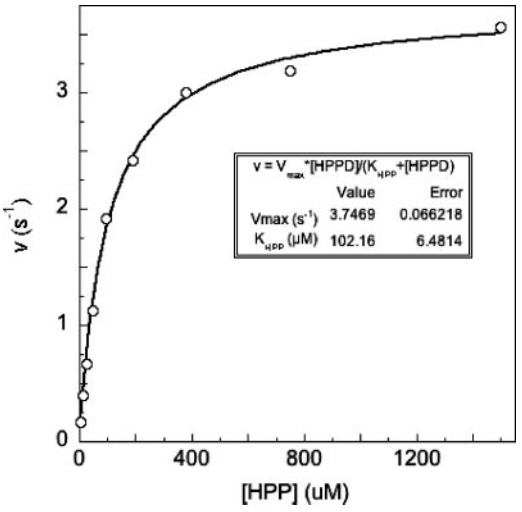

FIG. 4. Steady-state kinetic analysis of *B. cenocepacia* HppD. Assays were performed using a DW1 Hansatech Oxygraph oxygen electrode. The enzymatic reaction was initiated by the addition of HPP.

synthesized via an HGA intermediate. This was confirmed by culturing LEK47 in the presence of 0.5 mM HGA (Fig. 2B). Therefore, the genetic and chemical complementation of the *hppD* mutant support the notion that *B. cenocepacia* C5425 synthesizes melanin via an HGA intermediate and not a DOPA intermediate.

HGA can be identified in the culture supernatants of C5424, but not LEK47. The production of HGA was analyzed in the culture supernatants of C5424, LEK47, and LEK47(pKK29) by HPLC. Cultures of C5424, LEK47, and LEK47(pKK29) were grown in LB for 36 h at 37°C with shaking. By visual inspection, no pigment was observed in LEK47 cultures, while pigment was readily observed in C5424 and LEK47(pKK29). By HPLC analysis, high levels of HGA were present in C5424 culture supernatants compared to LEK47, where the HGA peak was almost at background level (Fig. 3). Introduction of pKK29 into LEK47 resulted in the detection of HGA in the culture supernatant at an intermediate level compared to LEK47 and C5424. HppD expression from the plasmid pKK29 is under the control of the constitutive *dhfr* promoter and not the HppD native promoter. This difference could perhaps account for the differences in HGA levels in the culture supernatants of C5424 and LEK47(pKK29). Commercially available HGA was used as a standard and gave a single peak at 3.50 min (Fig. 3A). A 3.49-min peak was observed for C5424, and a small peak was observed just above background at 3.40 min in the LEK47 sample. LEK47(pKK29) and LEK47 spiked with HGA gave peaks at 3.49 and 3.51 min, respectively. Additionally, the UV spectrum of the 3.50-min peak from the HGA standard had a maximum of 290 nm (Fig. 3B). The UV spectra of the HGA peaks from C5424, LEK47(pKK29), and LEK47 spiked with HGA also had maxima of approximately 290 nm. No absorption at 290 nm was observed in the 3.40-min peak from LEK47. These data demonstrate that the disruption of BCAL0207 is associated with the absence of HGA in the culture supernatant of the mutant LEK47 strain. This experiment strongly suggests that BCAL0207 encodes an HppD enzyme that catalyzes the conversion of 4-hydroxyphenylpyruvate to HGA in *B. cenocepacia*.

***B. cenocepacia hppD* encodes an active HppD enzyme.** *B. cenocepacia* C5424 *hppD* was amplified by PCR, cloned into pET28a, and transformed into *E. coli* BL21(DE3) to overexpress and purify the protein. Large quantities of soluble protein (approximately 90% of the HppD protein present in the cell) were observed in the cytoplasm after 4 h of induction with 0.5 mM IPTG. HppD was purified using metal ion affinity and then size exclusion chromatography (data not shown). The size exclusion chromatography profiles suggested that the *B. cenocepacia* HppD protein is present in a tetrameric confirmation, as described previously for *P. fluorescens* HppD (53). Steady-state kinetic parameters were determined for *B. cenocepacia* HppD using a Hansatech Oxygraph oxygen electrode, with reactions initiated by the addition of the HPP substrate. The V_{max} for *B. cenocepacia* HppD was determined to be 3.747 ± 0.07 s^{-1} and the K_m was 102.16 ± 6.5 μM (Fig. 4), with an estimated specific activity at 415 μM O_2 of 2 s^{-1}. This result correlates with the HPLC analysis of culture supernatants, confirming that BCAL0207 encodes an active HppD. The turnover rate of *B. cenocepacia* HppD is comparable to that described previously for HppD proteins from a range of organisms, including *S. avermitilus* and *Arabidopsis thaliana* (22, 41).

Loss of pigment production results in increased susceptibility to oxidative stress. Melanins have a strong affinity for metals and are highly effective scavengers of free radicals (55). *Cryptococcus neoformans* produces a melanin pigment that plays an important antioxidant function, with melanized cryp-

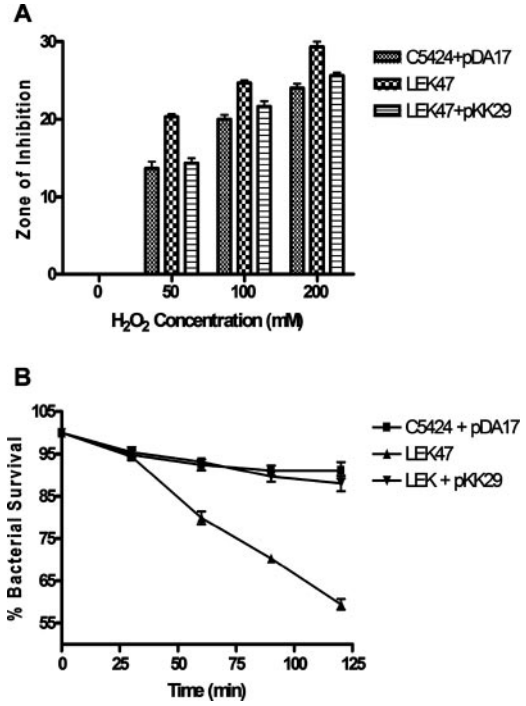

FIG. 5. LEK47 is susceptible to killing by hydrogen peroxide and extracellular O_2^-. (A) Susceptibilities to hydrogen peroxide of C5424(pDA17), LEK47, and LEK47(pKK29). The error bars represent standard deviations in the zones of inhibition obtained from triplicate plating at each concentration of hydrogen peroxide. (B) Resistance of *B. cenocepacia* C5424(pDA17), LEK47, and LEK47(pKK29) to killing by O_2^- generated by the xanthine/xanthine oxidase reaction. The error bars represent standard deviations in the percentage of surviving bacteria obtained from triplicate plating taken at each time point.

tococcal cells being more resistant to oxygen- and nitrogen-derived oxidants than nonmelanized cells (61). *Azotobacter chroococcum* produces melanin that has been associated with protection against reactive oxygen species (54), and iron binding by melanin in *Azotobacter salinestris* may protect the organism from damage caused by hydrogen peroxide (40). Production of pigment by group B *Streptococcus* also confers resistance to oxidative stresses, including H_2O_2 and superoxide (32). Production and characterization of a brown melanin pigment from *B. cenocepacia* strain P1 has previously been described, and it was demonstrated that the purified pigment can scavenge superoxide anions produced by a monocyte cell line upon activation with lipopolysaccharide, thus potentially aiding colonization and infection (64). The protection of LEK47 against H_2O_2 challenge was compared to that of the parental strain, C5424, and the complemented strain LEK47(pKK29) after incubation at 37°C for 36 h, a condition under which the parental strain was pigmented. When challenged with either 50, 100, or 200 mM of H_2O_2, LEK47 exhibited a larger zone of inhibition than the parental strain, and this defect could be complemented by the plasmid pKK29 (Fig. 5A). At all H_2O_2 concentrations tested in this study, LEK47 was approximately 23% more susceptible to H_2O_2 killing. LEK47 was investigated for sensitivity to extracellular superoxide generated by the xanthine/xanthine oxidase method. As previously described, 36-h cultures were used in the assay. After incubation with xanthine/xanthine oxidase for 2 h, LEK47 exhibited a decrease in bacterial survival of 40.5%, while the parental strain exhibited a decrease of 8.9% over the same time frame. The addition of the plasmid pKK29 rescued this phenotype to wild-type levels (Fig. 5B). When C5424 and LEK47 were challenged with H_2O_2 and superoxide after 24 h of incubation at 37°C, before C5424 was pigmented, there was no difference in the susceptibilities of LEK47 to H_2O_2 and superoxide (data not shown). LEK47 and C5424 were also tested for susceptibility to paraquat, which generates superoxide intracellularly, and no difference was detected. Melanin produced by *B. cenocepacia* is present in the culture supernatant and would be expected to exert its protective effect against extracellularly generated oxidative stress; therefore, it is not surprising that methyl viologen has no effect on LEK47.

LEK47 colocalizes with dextran-loaded phagosomal compartments. Work previously performed in our laboratory demonstrated that *B. cenocepacia*, in contrast to classical intracellular pathogens, survives intracellularly in a membrane-bound vacuole by a strategy that involves a delay in the phagolysosomal fusion but does not replicate in either amoebae or macrophages (27, 28, 34, 49). Microscopic single-cell analysis was used to determine the intracellular location of LEK47 compared to the parental strain, C5424, in prelabeled fluorescent cellular compartments. A RAW264.7 macrophage-like cell line was utilized to investigate whether the disruption of *hppD*, and therefore abrogation of pigment production in LEK47, affects intracellular localization. Experiments were performed to assess the colocalization of both C5424 and LEK47 with lysosomes that were preloaded with TMR-dextran (10,000 molecular weight). TMR-dextran is endocytized by macrophages and traffics through the endosomal pathway, accumulating in the lysosomes. Figure 6A shows that C5424 is phagocytized and resides within a spacious membrane-bound vacuole. At 4 h postinfection, 21.1% ± 2.3% of the *B. cenocepacia*-containing vacuoles colocalize with TMR-dextran. In contrast, at 4 h postinfection, 58.8% ± 1.8% ($P = <0.0001$) of the *B. cenocepacia* LEK47-containing vacuoles colocalize with TMR-dextran. DPI, an inhibitor of flavoproteins, including NADPH oxidase, or AG, an inhibitor of inducible nitric oxide synthase, was also added concurrently with LEK47, and the assay was repeated. At 4 h postinfection, 32.3% ± 2.5% ($P = 0.0001$) of the *B. cenocepacia* LEK47-containing vacuoles colocalized with TMR-dextran in DPI-treated cells and 44.3% ± 3.6% ($P = 0.0032$) of the *B. cenocepacia* LEK47-containing vacuoles colocalized with TMR-dextran in AG-treated cells (Fig. 6C.). These results suggest that the melanin species present in C5424 acts as both a superoxide radical scavenger (64) and a reactive nitrogen species scavenger, demonstrating the important role that the pigment plays in protecting *B. cenocepacia* from oxidative damage when it is phagocytized by macrophages. The absence of pigment in LEK47 appears to result in oxidative damage, and as a result of this oxidative damage, LEK47 is preferentially trafficked to a lysosomal compartment in the macrophage, where it is destroyed. Melanin plays an important role in the protection of a number of fungi during phagocytosis by both macrophages and neutrophils. Phagocytosis of pigmented *Exophiala dermatitidis* is not influenced by the presence of melanin; however, melanized cells are significantly

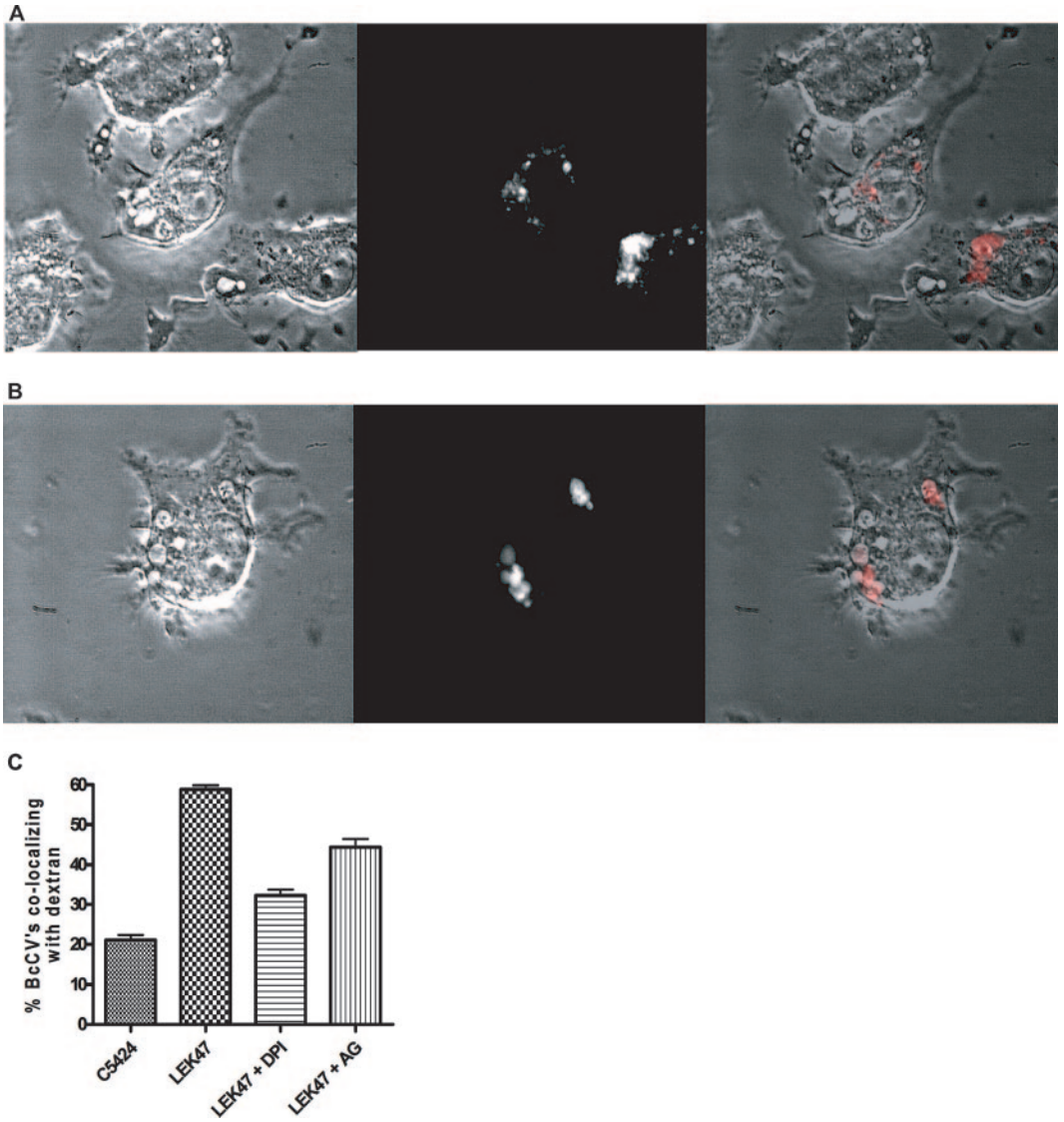

FIG. 6. LEK47 colocalizes with dextran-labeled lysosomes. A 2-h pulse-chase using TMR-dextran was performed to label the lysosomes, and then RAW 264.7 macrophages were infected for 4 h at a multiplicity of infection of 30 with either (A) C5424 or (B) LEK47. The macrophages were examined by fluorescence and phase-contrast microscopy. (C) The percentage of *B. cenocepacia*-containing vacuoles (BcCV's) colocalizing with TMR-dextran. The values represent the averages and standard deviations of three independent experiments in which 21 fields of view were examined at a magnification of ×100.

protected against killing (51). Cryptococcal melanization reduces phagocytosis and increases resistance to killing by macrophages, influencing the immune response to infection (60). Melanized *Sporothix schenchii* is also more resistant to phagocytosis and killing by murine macrophages than the nonpigmented strain (43).

A subset of *B. cenocepacia* strains produce pigment. A screen of 22 *B. cenocepacia* isolates from our collection (Table 2) revealed that only 4 strains produced pigment when cultured for up to 72 h on LB agar. BC7, J2315, C3865, and C5424 produced the characteristic dark-brown pigment after 36 h of incubation at 37°C on LB agar plates. The pigmented strains were isolated from CF patients in either Canada or the United Kingdom, and three of the strains, BC7, J2315, and C5424, belong to the ET-12 lineage. The ET-12 strain K56-2 does not produce pigment. BCAL0207 can be amplified by PCR from K56-2 genomic DNA, confirming that the gene is present in this strain. When pKK29 was conjugated into *B. cenocepacia* K56-2, it remained nonpigmented even after 48 h of incubation at 37°C; the expression of $HppD_{FLAG}$ was confirmed by Western blotting using an anti-FLAG antibody and was shown to be expressed in K56-2 (data not shown). K56-2 may not naturally produce pigment, as it could be defective in the conversion of tyrosine to 4-hydroxyphenylpyruvate. Thus, there would be no substrate for the HppD to act upon to produce HGA. Alternatively, K56-2 could efficiently convert any HGA produced into fumaric acid and acetoacetic acid via the action of homogentisic acid 1,2-dioxygenase, and these catabolites could be channeled into the Krebs cycle. This mechanism is currently under further investigation.

TABLE 2. Pigment production in *B. cenocepacia*

Strain	Pigment production	Source or reference[a]
C5424	Yes	BCRRC; CF isolate
K56-2	No	BCRRC; CF isolate
CEP024	No	CF isolate[b]
CEP511	No	BCRRC; CF isolate
C1484	No	CF isolate[b]
C3865	Yes	CF isolate[b]
C4455	No	CF isolate[b]
C6061	No	CF isolate[b]
H111	No	CF isolate
J2315	Yes	CF isolate; Edinburgh genome sequencing strain
CP 706-J	No	CF isolate; Cleveland
F28368-82	No	CF isolate; Toronto
L10	No	CF isolate; London
FC0127	No	CGD isolate[b]
F38192-89	No	CF isolate; Toronto
PC701-J	No	CF isolate; Cleveland
CEP0931	No	CGD isolate[b]
CEP1067	No	CGD isolate[b]
BC7	Yes	CF isolate; Canada
LMG19235	No	Lupine root isolate; Australia
LMG193239	No	Wheat pasture isolate; Australia
LMG21462	No	CF isolate; Italy

[a] BCRRC, *B. cepacia* Complex Research and Referral Repository for Canadian CF Clinics; CGD, chronic granulomatous disease.
[b] Strain provided by D. Speert, University of British Columbia.

It is unclear if there is a direct link between pigment production and increased levels of pathogenicity in *B. cenocepacia* infections. Three of the four pigmented strains identified in this study belong to the ET-12 lineage. ET-12 strains are multidrug-resistant bacteria and can be transmitted between CF patients, and CF patients infected with ET-12 clones have a fourfold increase in mortality compared to those patients infected with non-ET-12 clones (29). Pigment production has been shown in most fungi isolated from soil, including human-pathogenic fungi, and it has been postulated that the pigment may protect the organisms from diverse environmental stresses, including UV light (37). *B. cenocepacia* is also an environmental organism that can be readily isolated from soil. Therefore, it is possible that the primary role of the pigment produced by a number *B. cenocepacia* strains in specific environmental niches is to convey a selective advantage over neighboring organisms and that the pigment has a secondary "accidental role" in the protection of *B. cenocepacia* against reactive oxygen and reactive nitrogen species within a host organism.

Concluding remarks. This study demonstrates the identification and characterization of the *B. cenocepacia hppD* gene encoding an HppD and that *B. cenocepacia* C5424 produces a pigment using an HGA intermediate. We found that this melanin-like pigment plays an important role in protecting the organism from oxidative damage by host cells. Loss of pigment production resulted in the generation of a *B. cenocepacia* strain that was more sensitive to oxidative stress in vitro. The nonpigmented strain was also processed differently by macrophages, and unlike the wild-type strain, trafficked more readily to the lysosomal compartment. Thus, melanin production may be another factor contributing to increased levels of colonization and persistence in a subset of *B. cenocepacia* strains.

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